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14. ABSTRACT
BRCA1 C-terminal (BRCT) domains are novel phosphopeptide binding modules. Cancer-associated missense and deletion mutations have been found in the BRCT repeat regions of BRCA1, suggesting an essential role of BRCT domains in regulating BRCA1activity. In addition, BRCT domains are found in many proteins that regulate DNA damage repair, cell cycle, and genome stability, implying a more global role of BRCT domains in genome stability surveillance. These results suggest that the BRCT domain acts as a sensor to protein phosphorylation in response to DNA damage, recruits phosphorylated cellular targets, and mediates signaling complex formation. However, the identities of the in vivo BRCT domain targets are largely unknown. In order to understand the role of phosphorylation in protein-protein interactions, we developed several approaches utilizing peptide libraries and peptide arrays. We propose to use these methods to systematically identify phosphorproteins that can interact with BRCT domains. In addition to potential new regulators of genome stability, the approaches can identify phosphorylated sequences on proteins that are important for DNA damage responses and cell cycle. Such information should prove valuable, especially for the development of new screening strategies, drug targets, and treatment for breast cancer.

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BRCT domain, peptide library, OPAL, peptide array, proteomics, genome wide, signal transduction pathways, androgen receptor

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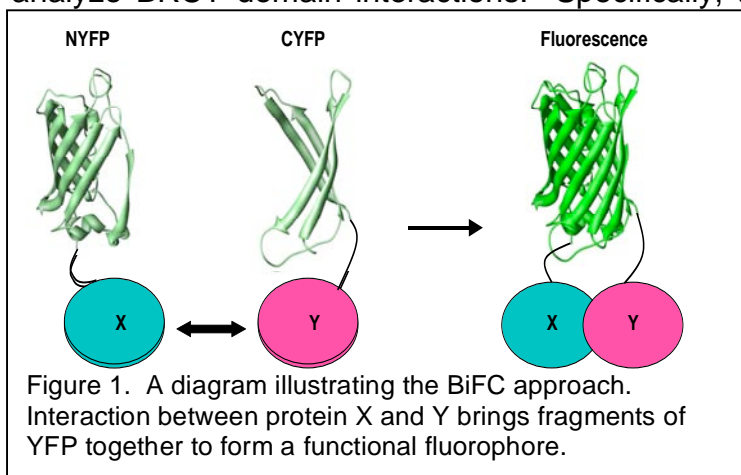Prescribed by ANSI Std. Z39.18

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Introduction

Understanding the molecular and cellular mechanisms that trigger breast cancer is essential to the prevention and treatment of this disease. The BRCA1 C-terminal (BRCT) domain was first identified in BRCA1 (1, 3). Cancer associated missense and deletion mutations have been found in the BRCT repeat regions of BRCA1, suggesting an important role of BRCT domains in regulating BRCA1 activity (8, 12). In addition, the BRCT domain is found in many proteins that regulate DNA damage repair, cell cycle, and genome stability, implying a more global role of BRCT domains in genome stability surveillance (1, 3). Consistent with this notion, the BRCT domain has been shown to mediate protein-protein interactions. For example, BRCT domains of BRCA1 associate with helicase BACH1 and CtBP interacting protein CTIP (4, 20). Recently, our lab and others have discovered that BRCT domains are novel phosphopeptide binding modules (10, 14, 19). BRCA1 BRCT domains associate with residue Ser990 on BACH1 in a phosphorylation-dependent manner. Furthermore, we found that several other BRCT domains including those from MDC1 and tumor suppressor BARD1 can bind specific phosphorylated peptides (6, 9, 15). These findings suggest that the BRCT domain recruits phosphorylated cellular targets and mediates signaling complex formation. However, the identities of the *in vivo* BRCT domain targets are largely unknown. In this application, we propose to systematically identify phosphoproteins that can interact with BRCT domains. Through these efforts, we may uncover potential new regulators of genome stability; more importantly, the approach can identify phosphorylated sequences on proteins that are important for DNA damage responses and cell cycle. Such information will help us to understand the mechanism of how protein phosphorylation modulates DNA damage responses and cell cycle in breast epithelial cells. In addition, it should prove invaluable for the development of new screening strategies and treatment for breast cancer.

Body

A. For Task 1, we proposed to identify phosphorylated peptide sequences that could specifically bind BRCA1 and BARD1 BRCT domains.

1.1 In our previous reports, we discussed technical problems in using OPAL arrays to analyze BRCT domain interactions. Specifically, the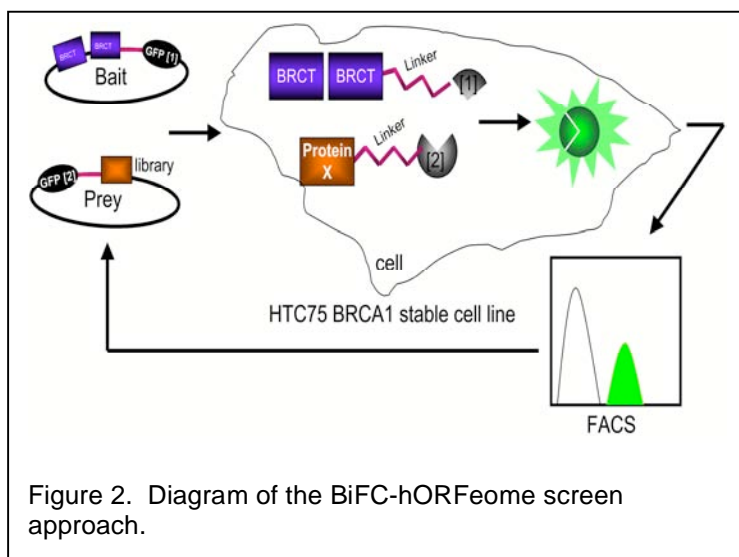re was high non-specific background due to surface chemistry. To circumvent these problems, we proposed to employ the alternative method -- Bi-molecular Flurescence Complementation (BiFC) -- to identify binding targets for BRCA1 and BARD1 and BRCT domains *in vivo*.

Figure 1. A diagram illustrating the BiFC approach. Interaction between protein X and Y brings fragments of YFP together to form a functional fluorophore.

Bimolecular fluorescence complementation (BiFC) was originally developed to visualize protein-protein interactions in live cells (7). Two

separate FP fragments (the N- or C-terminal half of a fluorescence protein such as YFP) are respectively fused to two proteins of interest. If the two proteins interact, the YFP fragments will be brought to close proximity and form a functional YFP protein complex (Figure 1).

BiFC offers several advantages for establishing protein-protein interactions. For example, it enables the detection of interactions *in vivo*, allows for the visualization of the subcellular locations of specific protein-protein interactions, and it is highly amenable to investigating inducible interactions including DNA damage induced interactions.

Figure 2. Diagram of the BiFC-hORFeome screen approach.

(1) BiFC-hORFeome design to screen for BRCA1 and BRCT targets

To develop the BiFC technology for screening for BRCT-BRCA1 interacting proteins in mammalian cells, we constructed expression vectors using the Gateway® cloning system. These vectors were designed to encode either the N- or C-terminal half of YFP (YFPn and YFPc respectively). For the bait, we engineered BRCA1 BRCT domain sequences tagged by YFPn and established stable cell lines expressing YFPn-BRCT.

We reported previously the generation of YFPc-tagged cDNA libraries from the Human Open Reading Frame Collection (hORFeome, Openbiosystems). The hORFeome contains ~8,000 human individual open reading frames and was used as prey in our studies (Figure 2). Since the last report, we have improved upon the BiFC-hORFeome library by constructing a new and more comprehensive hORFeome library that now contains ~12,000 human individual open reading frames. This 1/3 increase in genome coverage should allow us to more thoroughly screen for BRCT interacting partners.

(2) BiFC-hORFeome screens for BRCT domain

To screen for BRCA1-BRCT interacting proteins using BiFC, we undertook several approaches. In the last report, we described a reduction approach where the ~8,000 hORFeome library was divided into pools. Each pool was then used to generate high-titer retroviruses for subsequent infection of the YFPn-tagged BRCT-BRCA1 expressing stable cell line. Interaction between YFPn-BRCA1-BRCT and YFPc-tagged prey proteins would bring YFPn and YFPc to close proximity and allow for the detection of YFP+ cells/pools. After three rounds of further division and enrichment, we have narrowed down the number of BRCT-BRCA1 candidate binders from ~8,000 genes to ~150 genes. These ~150 candidates are involved in diverse cellular functions including ubiquitination, chromosome maintenance, and cell cycle control.
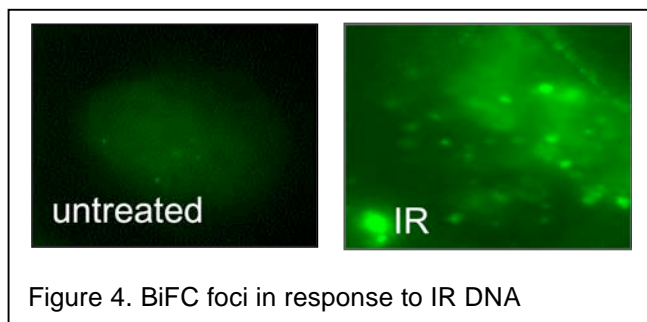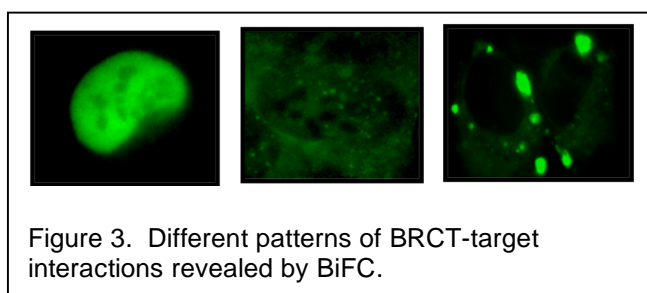
(2.1) FACS sorting screen

In the process of performing the Reduction pooling screen, we encountered many of the limitations of this approach. For example, it biased toward strong protein-protein interactions, because such interactions would tend to give rise to higher YFP fluorescence intensity, and are therefore disproportionately enriched in subsequent steps. In addition, the reduction approach takes a much longer time to identify the clones that interact with BRCA1 BRCT domain.

We therefore carried out an alternative method. After infecting the YFPn-tagged BRCT-BRCA1 expressing stable cell line using high-titer 8,000 hORFeome retroviruses, the cells were individually sorted by FACS. Single YFP positive cells were sorted individually into 96-well plates. After the cells recovered and expanded, they were further confirmed for whether they remained YFP positive. Genomic DNA was then extracted from the positive clones and used as PCR templates to identify the candidate BRCT-interacting proteins.

Indeed, we obtained many cell clones that exhibited either strong or weak fluorescence, indicating that these cells may express potential BRCA1 interacting proteins. We then carried out PCR and sequencing analysis of these isolated clones. Among the genes identified through this approach, several factors were previously unsuspected in DNA damage pathways, for example, NACAP1 and NOL5A. Interestingly, some of these proteins were also in the short list of genes identified from our reduction pooling screens, suggesting that these proteins may indeed be true interactors of BRCA1.

(2.3) To determine where interactions occur and whether interactions are responsive to DNA damage signaling

Figure 3. Different patterns of BRCT-target interactions revealed by BiFC.

Figure 4. BiFC foci in response to IR DNA

Next, we carried out experiments to further investigate these putative interactors. First, we analyzed the sub-cellular localization of YFP signals in these cells under fluorescence microscope. We found that the interactions occurred in distinct subcellular compartments amongst different clones (Figure 3). Such findings indicate that (1) the BiFC-hORFeome approach is capable of identifying interactions in different subcellular locations; and (2) the BiFC-hORFeome approach is capable of identifying different types of interactors.

Because BRCA1 mediates DNA damage response, we reasoned that BRCA1-target interaction may be regulated by DNA damage. It has been demonstrated that the BRCT motif is important for BRCA1 nuclear localization (nuclear foci) during S phase and its recruitment to double-stranded break (DSB) foci after irradiation (IR). These foci likely represent sites of DNA damage. We therefore determined whether the localization of the YFP signal (which indicates where the interactions occur) was altered after IR. As shown in Figure 4, we found that some of the putative BRCA1 interacting proteins formed foci after IR treatment, indicating that they may interact with BRCA1 BRCT domain and participate in DNA damage response.

1.2   During the past year, we have also carried out biochemical and structural studies of BRCT domains and their targets.

BRCT domains	pSer pocket		P+3 pocket			Known Motif or peptides
	Lβ1α1	α2	Lβ1'α1'	α2	α3'	
BRCA1	SG	K	M	F	L	pSPTF
MDC1	TG	K	P	L	L	pSQEY
MCPH1	SG	N	P	L	L	pSQEY
BARD1	SG	K	H	M	I	pSEDE?
ECT2	TG	K	E	R	W	?
PTIP (560-757)	TG	K	P	L	L	pSQVF pSQEY?
TOPBP1 (22-207)	TS	K	L	L	F	?
TOPBP1 (1177-1401)	SS	K	E	L	A	?
XRCC1	SG	K	E	S	Y	?
BRCTD1	TG	K	K	L	G	?
DNL4	SG	K	T	I	T	pSYYI?

Figure 5. .   Predicted phosphopeptide-binding pockets for BRCT

It remains unclear whether all BRCT domains can mediate phosphorylation-dependent interactions. We decided to use structural analyses to predict BRCT-phosphopeptide interactions. As evidenced by numerous crystal structures of BRCT domains, phosphopeptide recognition is achieved primarily through two key binding pockets formed by the tandem BRCT domains. The phosphoserine recognition pocket is formed by three residues on Lbeta1alpha1 and alpha2 from the first BRCT domain (Figure 5) (2, 5, 16, 17). All the BRCT repeats known to bind phosphopeptides contain a (T/S)G motif and a K/N residue within Lbeta1alpha1 and alpha2 respectively. Based on these observations, we have predicted 5 additional putative phosphopeptide-binding BRCT repeats from human BRCTD1, TOPBP1, ECT2, and XRCC1. These BRCT repeats harbor either the (T/S)G or a closely related (T/S)S motif at the corresponding Lbeta1alpha1 positions (Figure 5). These proteins are involved in cell cycle and DNA damage response. The identification of these putative phospho-binding BRCT motifs provides additional avenues of research into their function in cell cycle control and DNA damage response.

The other key-binding pocket is involved in specificity determination of BRCT-phosphopeptide interaction. As revealed by the structures of phosphopeptides binding to BRCA1 or MDC1 BRCT domains, the P+3 residue (relative to pSer) plays an important role in governing the specificity of BRCT repeats (2, 5, 16, 17). BRCA1 and MDC1 prefer Phe and Tyr respectively at this position (13). Unlike the phosphoserine-binding pocket that is mainly formed by residues from the first BRCT domain, the P+3 pocket is formed by residues from both the first and second BRCT domains (Figure 5). In the BRCA1 BRCT structure, the Phe residue from alpha2, Met residue from Lbeta1'alpha1', and Leu residue from alpha3' contribute to Phe recognition at the pSer+3 position. In comparison, Leu of alpha2, Pro of Lbeta1'alpha1', and Leu of alpha3' help to coordinate the recognition of Tyr at the P+3 position in the MDC1-H2AX peptide structure. Interestingly, MCPH1 contains the same residues as MDC1 in the P+3 pocket and was shown recently to bind the phospho-H2AX peptide (18). These data lend support to utilizing the residues that make up the pSer+3 binding pocket for specificity prediction of BRCT domains. For example, the P+3 pocket residues from PTIP BRCT repeats (residues 560-757) are similar to those of MDC1.

Accordingly, PTIP was shown to bind with high affinity peptides with Phe at the P+3 position (11). It is also possible that PTIP BRCT domains may interact with the phosphorylated tail of H2AX (Figure 5). This may explain the finding that PTIP is targeted to phospho-H2AX DNA damage foci (11).

These findings and results have been included in a manuscript *in press* in Frontiers in Biosciences.

B. For Task 2, we proposed to characterize breast cancer genes and BRCT binding sites identified in Task 1, by RNAi and by determining the role of BRCT-phosphopeptide interaction in DNA damage.

We have identified several potential interacting proteins of BRCA1 BRCT domain. While we are confirming the interaction of these proteins with BRCA1, we have obtained lentiviral shRNA vectors for these potential targets. We will address whether knocking down these putative targets in human cells could inhibit or potentiate IR-induced DNA damage response. Furthermore, in cells where expression of these putative targets was inhibited, whether their cell cycle profiles are altered will also be examined.

Key Research Accomplishments

- We have carried out several BiFC *in vivo* interaction studies and obtained a number of novel interaction partners for BRCA1-BRCT domains.
- We have demonstrated that localization of the interaction between BRCA1 and its partner is regulated by DNA damage such as IR in live cells.
- We have performed structural analyses on BRCT domains and found several BRCT domains capable of binding to phosphopetide.
- We have developed a strategy to predict binding specificities of BRCT domains.

Reportable Outcomes

Manuscript in press
BRCT domains: phosphopeptide binding and signaling modules. (2008) Maria Rodriguez, Zhou Songyang. *Frontiers in Biosciences, in press*

Conclusions

In summary, we have sucessefully conducted genetic screens of BRCT domain interacting sequences using BiFC. We have obtained and confirmed a number of potential targets for further examination. We have performed structural analyses on BRCT domains and predict BRCT domain-phosphopeptide interactions. The information obtained from our studies should prove especially useful for the development of new and effective screening strategies, drug targets, and treatment for breast cancer.

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